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Lymphocyte Proliferation Response During *Eimeria tenella* Infection Assessed by a New, Reliable, Nonradioactive Colorimetric Assay

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SUMMARY. The application of a tetrazolium salt, WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt to the lymphocyte proliferation assay in the chicken system was evaluated. Proliferation of concanavalin (Con A)-induced splenic lymphocytes and peripheral blood lymphocytes (PBL) was evaluated with WST-8 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Coefficients of correlation (*r*) between these two reagents were 0.98 and 0.97 in splenic lymphocytes and PBL, respectively. In general, the sensitivity of the WST-8 assay was significantly higher than that of the MTT assay, and the standard deviations of the WST-8 assay were significantly lower than those of the MTT assay. The WST-8 assay was fast and highly reproducible and provided a good indication of mitogen-induced proliferation of spleen cells induced by Con A. With the use of the WST-8 assay, splenic mitogenic response of chickens infected with *Eimeria* decreased transiently at 7 days but increased significantly at 10 days after primary infection compared with that of uninfected chickens. Additionally, the measurement of interleukin (IL)-2 production with WST-8 was highly reproducible and showed a significant increase in IL-2 production upon stimulation of *Eimeria tenella*-immune spleen cells with Con A. After *E. tenella* infection, splenic IL-2 production increased significantly at 7 days post-primary and at 2 days post-secondary infection. The WST-8 assay is fast, simple, and more reproducible and sensitive than the MTT assay. This study demonstrates the effectiveness of the WST-8 assay to assess cell-mediated immune response of chickens in normal and disease states.

RESUMEN. Respuesta proliferativa de linfocitos durante la infección con Eimeria tenella, determinada por un nuevo método colorimétrico no radiactivo y confiable.

Se evaluó la aplicación de sales de tetrazolio (WST-8) en el ensayo de proliferación linfocitaria para pollos. Se evaluó la proliferación de linfocitos esplénicos y linfocitos de sangre periférica inducida con concanavalina A usando WST-8 y dimetiltiazol bromuro de difeniltetrazolio (MTT). Los coeficientes de correlación (r) entre los dos agentes fueron de 0.98 y 0.97 para linfocitos esplénicos y linfocitos de sangre periférica, respectivamente. En general, la sensibilidad del ensayo con WST-8 fue significativamente mayor que la del ensayo con MTT y las desviaciones estándar del ensayo con WST-8 fueron significativamente menores que aquellas con el ensayo con MTT. El ensayo con WST-8 fue rápido y altamente reproducible y aportó buena indicación de proliferación de células esplénicas inducidas por concanavalina A. Con el uso del ensayo de WST-8, la respuesta mitogénica de células esplénicas infectadas con *Eimeria* disminuyó transitoriamente a los 7 días pero aumentó significativamente a los 10 días después de la infección primaria en comparación con los pollos no infectados. Adicionalmente, las mediciones de producción de interleucina (IL)-2 con WST-8 fueron altamente reproducibles y se observó un aumento significativo en la producción de IL-2 después de la estimulación con concanavalina A de células esplénicas inmunes a *E. tenella.* Después de la infección con *E. tenella,* la producción en bazo de IL-2 aumentó significativamente a los 7 días después de la infección primaria y dos días después de la infección secundaria. El ensayo con WST-8 es rápido, simple y con mayor reproducibilidad y sensibilidad que el ensayo con MTT. Este estudio demostró la efectividad del ensayo con

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WST-8 para determinar la respuesta inmune celular de pollos bajo condiciones normales y de enfermedad.

Key words: WST-8, lymphocyte proliferation, chickens, IL-2 assay, *Eimeria tenella*

Abbreviations: CMI = cell-mediated immunity; Con A = concanavalin A; ELISA = enzyme-linked immunosorbent assay; HBSS = Hanks balanced salt solution; IL = interleukin; IMDM = Iscove modified Dulbecco medium; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD = optical density; PBL = peripheral blood lymphocytes; PMS = 1-methoxy-5-methylphenazinium methylsulfate; PPI = post-primary infection; PSI = post-secondary infection; SI = stimulation index; WST-8 = 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

Because of increasing evidence suggesting a role of cell-mediated immunity (CMI) in disease resistance against many poultry diseases (11,21), the lymphocyte proliferation assay is widely used to evaluate CMI in normal and disease states in chickens (12,13,15). A number of *in vitro* assays have been described for the evaluation of lymphocyte proliferation. Although the conventional [^{3}H]-thymidine incorporation assay is still considered the standard for the assessment of cell growth because of its sensitivity, reliability, and dynamic range (wide range counts per minute), the use of this assay has been restricted in recent years because it possesses a biological hazard to personnel and the environment, requires a separate facility and special equipment, and is labor intensive, time consuming, and expensive (2,3,19). Therefore, in order to circumvent the disadvantages of using [^{3}H]-thymidine in proliferation assays, several nonradioactive alternatives have been described in mammalian systems.

A nonradioisotopic, colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) has been accepted as a desirable assay for both mammals and poultry because it is fast, cost efficient, and safe (1,3,14,20). A comparative evaluation between the MTT assay and [^{3}H]-thymidine incorporation assay for lymphocyte proliferation in chickens (1,3) demonstrated a close correlation between these two assays, indicating that the MTT assay is suitable to use for evaluation of lymphocyte proliferation in chickens. However, the MTT assay has many drawbacks. Metabolism of the yellow tetrazolium salt MTT by mitochondrial dehydrogenases of dividing cells forms insoluble blue formazan crystals, which need to be dissolved before spectrophotometric measurement. This step increases sample processing time and can be a potential source of error.

Recently, a new tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), has been applied to the assessment of cell viability and lymphocyte proliferation in mammalian systems (8,18). The WST-8 produces a highly water-soluble formazan dye upon biochemical reduction in the presence of an electron carrier, 1-methoxy-5-methylphenazinium methylsulfate (PMS). The WST-8 assay also requires no radioisotope nor any organic solvent to dissolve the generated formazan dye, unlike the MTT, because of its high water solubility. The amount of formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture medium.

The purpose of the present study was to evaluate the application of the WST-8 to the chicken lymphocyte proliferation assay and to measure mitogen-induced proliferation to monitor the kinetic changes of CMI after *Eimeria tenella* infection.

MATERIALS AND METHODS

Chickens, parasite, and experimental infections. Specific-pathogen-free white leghorn chickens from Hyline International Production Center (Dallas Center, IA) were obtained as fertile eggs, hatched at the Animal and Natural Resources Institute facilities, Agricultural Research Service, USDA. Chickens were kept in wire cages and provided feed and water *ad libitum* with a constant light source. The strain of *E. tenella* used was developed by single oocyst isolation from wild-type *E. tenella* originally from Wisconsin and maintained at the Parasite Biology, Epidemiology, and Systematics Laboratory. For proliferation assays, splenic lymphocytes and peripheral blood lymphocytes (PBL) obtained from 6-wk-old chickens

were used. To evaluate CMI of chickens after *E. tenella* infection, 60 chickens were separated into two groups as infected and uninfected groups. The chickens were inoculated esophageally with 1×10^4 sporulated oocysts at 3 wk of age and subsequently given a secondary infection with 1×10^5 oocysts on day 21 after primary infection.

Preparation of splenic lymphocytes and PBL. Whole blood was used as the source of PBL. Blood was collected by cardiac puncture with a syringe containing heparin (20 units/ml blood) (Elkins-Sinn, Inc., Cherry Hill, NJ). Each blood sample was diluted with the same amount of Hanks balanced salt solution (HBSS). Spleens were macerated with a syringe plunger through a screen sieve to obtain a single-cell suspension in HBSS. Cell suspensions were overlaid onto Histopaque® 1077 density gradient medium and centrifuged at 1800 rpm for 20 min at room temperature. Lymphocytes at the interface were collected and washed three times in HBSS, and viable cells were counted by the trypan blue dye exclusion assay.

Lymphocyte proliferation assay. Serial dilutions of splenic lymphocytes and PBL were prepared in Iscove modified Dulbecco medium (IMDM) (Life Technologies Inc., Grand Island, NY) containing 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 2×10^{-6} M 2-mercaptoethanol, 5 μg 5-fluorocytosine/ml, and 1 mM sodium pyruvate (IMDM-10), and 100 μl of cells was put into each well of a 96-well flat-bottomed tissue culture plate (Corning Incorporated, Corning, NY). The final concentrations of cells used were 5×10^6, 2.5×10^6, 1.25×10^6, 6.25×10^5, 3.13×10^5, 1.56×10^5, and 7.81×10^4 cells/well. One hundred microliters of concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) at a final concentration of 50, 25, 12.5, or 6.25 μg/ml was added to each well. Medium without Con A was used as a negative control. Each assay was performed in triplicate. Plates were incubated at 41 C for 24 or 48 hr in a 5% CO_2, 95% humidity incubator.

The MTT assay was performed as described previously (3,7). The MTT solution was prepared by dissolving 10 mg MTT/ml calcium- and magnesium-free phosphate-buffered saline and sterilized by filtering through a 45-μm syringe filter. For the MTT assay, 20 μl of MTT solution was added to each well, and the plates were incubated at 41 C for 3 hr in a 5% CO_2 incubator. The plates were centrifuged at $1000 \times g$ for 10 min at room temperature, the supernatant was carefully removed, and 150 μl of a 10% saponin (Fisher Scientific, Fair Lawn, NJ) solution was added to lyse the cells as described (6). The plates were shaken for 20 min, the cells were thoroughly resuspended by multiple pipetting and centrifuged at $1000 \times g$ for 10 min, and the supernatant was removed. To dissolve the formazan crystals, 175 μl of 0.02 M HCl in isopropanol was added, the plates were shaken, and the pellets were thoroughly resuspended with a pipetter. The optical density (OD) of each well was measured at 550 nm with an enzyme-linked immunosorbent assay (ELISA) microtiter plate reader, model 3550 (Bio-Rad Laboratories, Hercules, CA). The WST assay was performed according to the manufacturer's instructions. The WST-8 working solution (Cell counting kit-8®; Dojindo Molecular Technologies, Inc., Gaithersburg, MD) contained WST-8 and 1-methoxy PMS (0.5 mM and 20 μM, respectively, as the final concentrations). After incubation, 10 μl of WST-8 working solution was added to each well, and the plates were incubated for 4 hr. The OD of each well was measured at 450 nm with the ELISA microtiter plate reader. Stimulation index (SI) was calculated by the following formula: SI = mean OD of Con A–stimulated cells/mean OD of unstimulated cells.

Quantification of chicken interleukin (IL)-2 levels in Con A–stimulated splenic lymphocyte culture supernatant. IL-2 levels in the culture supernatant of Con A–stimulated splenic lymphocytes after *E. tenella* infection were quantified by the bioassay (13). Spleen cells were adjusted to 5×10^6 cells/ml and incubated in IMDM-10 containing Con A (12.5 μg/ml) at 41 C in 5% CO_2, and the culture supernatants were harvested at 48 hr after incubation.

To prepare the blast cells for IL-2 bioassay, splenic lymphocytes were resuspended at 5×10^6 cells/ml in IMDM-10 with Con A (12.5 μg/ml) and incubated at 41 C in 5% CO_2 for 48 hr. After incubation, dead cells were removed by centrifugation through Histopaque-1077® density gradient medium, and viable cells were treated with 0.05 M α-methyl-mannoside. Blast cells (5×10^6 cells/ml) were resuspended in RPMI supplemented with 0.5% bovine serum albumin, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 2×10^{-6} M 2-mercaptoethanol, 5 μg 5-fluorocytosine/ml, and 1 mM sodium pyruvate; 100 μl of blast cells was dispensed into a 96-well flat-bottomed tissue culture plate, and the samples to be tested for IL-2 activity were added to each well at 100 μl/well. The mixture was incubated at 41 C for 48 hr. After incubation, proliferation was measured by the WST-8 assay as described above.

Statistical analysis. The statistical analysis to test the relation between MTT and WST-8 assays was performed as regression analysis. Significant differences between the infected group and uninfected group in lymphocyte proliferation assay and IL-2 bioassay were analyzed by *t*-test (GraphPad Instat, Version 3.0).

RESULTS

Assessment of splenic lymphocyte proliferation. A level of Con A (12.5 μg/ml) was

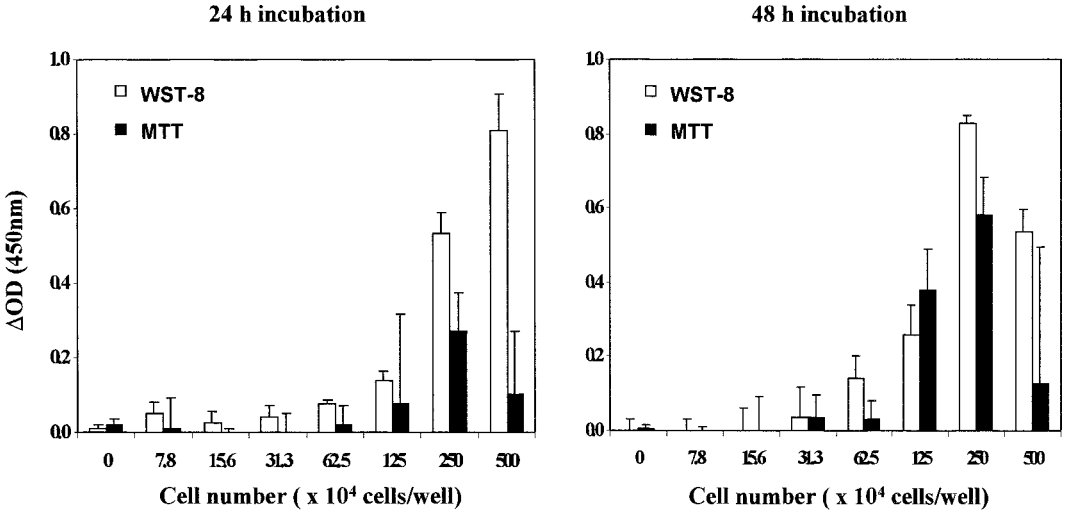

Fig. 1. Comparison of the MTT and WST-8 assays for measuring splenic lymphocyte proliferation. Various numbers of splenic lymphocytes were incubated in the presence or absence of Con A (12.5 μg/ml) for 24 or 48 hr. The proliferation was assessed by the MTT or the WST-8 assay. ΔOD = (mean OD of Con A–stimulated cells) - (mean OD of unstimulated cells). Each data point represents OD of spleen samples pooled from three chickens. Representative results from three independent studies are shown.

determined to be optimum for comparison of splenic lymphocyte proliferation by MTT and WST-8 assays (unpubl. obs.). The results of the proliferation of splenic lymphocytes with Con A are shown in Fig. 1. The pattern of proliferation response as assessed by the WST-8 assay closely resembled that derived from the MTT assay. A highly significant ($P < 0.001$) and linear relationship between MTT and WST-8 assays was observed (data not shown), and the coefficient of correlation (r) between these two assays was 0.98. The results showed that OD values in the Con A–stimulated cells were 1.2–2.9 times higher than those in the unstimulated cells and were highly proportional to the cell number by both the MTT and the WST-8 assays (Fig. 1). OD values of the WST-8 assay were higher than those of the MTT assay. The optimal conditions of splenic lymphocytes in the WST-8 assay were obtained when 2.5×10^6 cells/well were stimulated with Con A (12.5 μg/ml) for 48 hr.

Assessment of PBL proliferation. A level of Con A (12.5 μg/ml) was also determined to be optimum for the proliferation of PBL. The results of the proliferation of PBL with Con A (12.5 μg/ml) are shown in Fig. 2. The relationship between the MTT and the WST-8 assays was highly significant ($P < 0.001$) and showed a significant correlation (data not shown). The coefficient of correlation (r) between the MTT and the WST-8 assays was 0.97. OD values of PBL were significantly lower than those of splenic lymphocytes, although OD values were proportional to the cell numbers in both the MTT and the WST-8 assays. OD values of the Con A–stimulated PBL were 1.1–1.9 times higher than those of the unstimulated cells (Fig. 2). OD values of PBL in the WST-8 assay were significantly higher than those in the MTT assay. The optimal mitogenic response of PBL in the WST-8 assay was obtained when 1.25×10^6 cells/well was activated with Con A (12.5 μg/ml) for 48 hr.

Assessment of mitogenic response of splenic lymphocytes after *E. tenella* infection. Because optimal conditions for splenic lymphoproliferation were observed when 2.5×10^6 cells/well was cultured with Con A (12.5 μg/ml) for 48 hr, these conditions were used in the following studies. Fig. 3 shows the kinetic changes of Con A–stimulated splenic lymphocyte proliferation after inoculation with *E. tenella* as measured by the WST-8 assay. The SIs on day 7 post-primary infection (PPI) in the infected group were significantly ($P < 0.05$) lower but those on days 10 and 14 PPI in the infected group was significantly ($P < 0.05$)

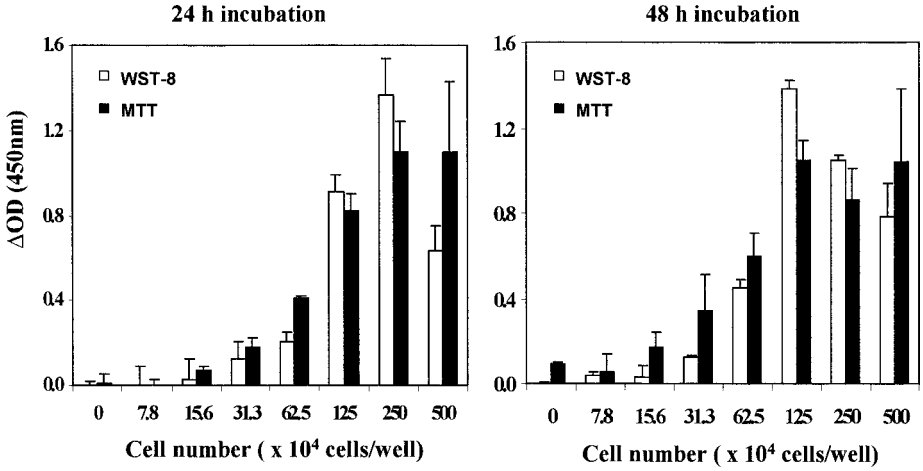

Fig. 2. Comparison of MTT and WST-8 assays for measuring proliferation of PBL. Various numbers of PBL were incubated in the presence or absence of Con A (12.5 μg/ml) for 24 or 48 hr. The proliferation was assessed by MTT or WST-8 assay. ΔOD = (mean OD of Con A–stimulated cells) − (mean OD of unstimulated cells). Each data point represents OD of PBL samples pooled from three chickens. Representative results from three independent studies are shown.

higher than those in the uninfected group (Fig. 3). The SIs of the infected chickens remained higher than those of the uninfected chickens after secondary infection. Proliferation in response to medium alone (background proliferation) from the infected chickens was significantly ($P < 0.05$) higher than that from the uninfected chickens on day 7 PPI (data not shown). Proliferation response to Con A from the infected chickens was higher than that from the uninfected chickens throughout the experimental period except on day 2 PPI (data not shown). The difference between the two groups was significant ($P < 0.01$) on day 10 PPI.

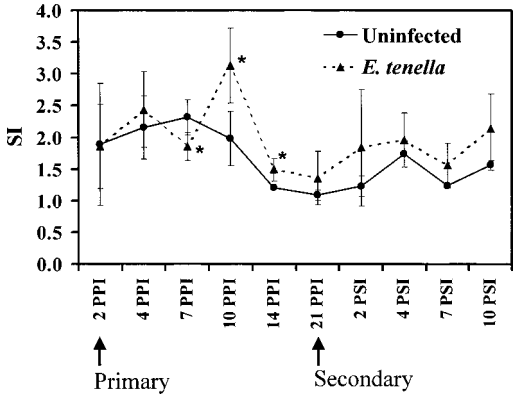

Fig. 3. The kinetic changes in Con A–induced splenic lymphocyte proliferation after *E. tenella* infection by the WST-8 assay. Medium or Con A was used to stimulate lymphocytes and stimulation indices (SIs) were calculated as described in Materials and Methods. The chickens in the infected group were inoculated esophageally with 1×10^4 sporulated oocysts of *E. tenella* at 3 wk of age and subsequently challenged with 1×10^5 oocysts on day 21 PPI. The data are expressed as mean SI ± SD. An asterisk indicates significant difference when compared with the uninfected group (*$P < 0.05$).

Changes of the IL-2 levels in Con A–stimulated splenic lymphocyte culture supernatant after *E. tenella* infection. Fig. 4 shows the kinetic changes in Con A–induced IL-2 production in the spleen after *E. tenella* infection as measured by the IL-2 bioassay with WST-8. The IL-2 levels on day 7 PPI and on day 2 post-secondary infection (PSI) in the infected group increased transiently. The IL-2 level in the infected group on day 10 PPI was significantly ($P < 0.05$) lower than that in the uninfected group.

DISCUSSION

Ishiyama *et al.* (8) have demonstrated that WST-8 has great potential as a viability indicator in cell proliferation or cytotoxicity assays. The advantages of using the WST-8 assay for lymphocyte proliferation are numerous. Unlike

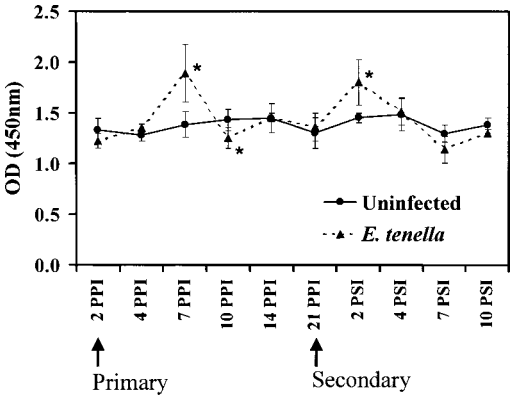

Fig. 4. The kinetic changes of IL-2 production after *E. tenella* infection as measured by the WST-8 assay. The chickens in the infected group were inoculated esophageally with 1×10^4 sporulated oocysts at 3 wk of age and subsequently challenged with 1×10^5 oocysts on day 21 PPI. The data are expressed as mean OD ± SD. An asterisk indicates significant difference when compared with the uninfected group ($^*P < 0.05$).

the MTT, the WST-8 assay does not require any dissolving step before measuring the absorbance because the WST-8 produces a highly water-soluble formazan dye upon biochemical reduction in living cells. The WST-8 assay obviates the concerns related to radioactivity. Moreover, because this reagent is supplied as a ready-to-use solution, no dilution with buffer or culture medium is necessary prior to use. Therefore, the WST-8 assay is quite simple, does not use radioactive isotope and can save considerable time. Furthermore, the WST-8 is stable over 1 yr at −20 C and over 3 mo at 4 C as long as it is protected from light. The color change is relatively stable; therefore, no absorbance change was observed within 48 hr after addition of 10 μl of 1% sodium dodecyl sulfate to each well as long as the plates were protected from light and stored at room temperature.

In the present experiments, the WST-8 assay was compared with the MTT assay on mitogen-induced lymphoproliferation of splenic lymphocytes and PBL. The coefficients of correlation between these assays were very high in both splenic lymphocytes and PBL. The sensitivity of the WST-8 assay was higher than that of the MTT assay. Additionally, the SDs of the WST-8 assay were lower than those of the MTT assay. Although the MTT assay has been reported to be effective for the evaluation of mitogen-induced proliferation with chicken lymphocytes (3), our data strongly suggested that the WST-8 assay was a more reliable measure of the proliferation of chicken lymphocytes. In the present experiments, the optimal conditions for splenic lymphocytes and PBL when using 6-wk-old chickens in the WST-8 assay were similar to those in the MTT assay and were obtained by culturing 2.5×10^6 cells/well with Con A (12.5 μg/ml) for 48 hr and 1.25×10^6 cells/well with Con A (12.5 μg/ml) for 48 hr, respectively.

Mitogen-induced lymphocyte proliferation has been used to assess CMI after coccidia infection in chickens (5,9,12,16). In general, the Con A–induced mitogenic response decreased around 7 days post-*E. tenella* infection (5,9,16) but increased after 2 wk of primary infection. The present experiments showed that the SIs of the infected chickens declined transiently on day 7 but were enhanced on day 10 PPI, consistent with previous observations. Furthermore, this study showed higher background proliferation of spleen cells from the infected chickens on day 7 PPI, which was consistent with a previous observation (4). These results suggested that WST-8 assay can effectively monitor the changes in T-lymphocyte response in chickens infected with *Eimeria* parasites.

In the mammalian system, IL-2 has long been detected with the IL-2–dependent T-cell line (e.g., CTLL line) that constitutively expresses the IL-2 receptor (7). On the other hand, because murine lymphocytes are unresponsive to crude chicken IL-2 (17) and no avian IL-2–dependent cell line is available, the bioassay for avian IL-2 has relied on T lymphoblasts generated by Con A stimulation (10,13,15). The MTT assay or [^{3}H]-thymidine incorporation assay has been used widely to determine the lymphocyte proliferation in poultry as in mammals. In this study, the IL-2 bioassay with the WST-8 clearly demonstrated the kinetic changes in IL-2 production by Con A–stimulated spleen cells from *E. tenella*-infected chickens. Maximum IL-2 production was observed on days 7 PPI and 2 PSI, respectively. These results indicate that chickens develop enhanced CMI against *E. tenella* after challenge infection.

In conclusion, this study shows that the WST-8 assay is more reliable and easier to process compared with the MTT assay for evaluation of lymphocyte proliferation in chickens.

REFERENCES

1. Agrawal, P. K., and D. L. Reynolds. An evaluation of the mitogenic reactivity of intestinal intraepithelial lymphocytes of chickens. Avian Dis. 43: 172–181. 1999.

2. Ahmed, S. A., R. M. Gogal, and J. E. Walsh. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H] thymidine incorporation assay. J. Immunol. Methods 170:211–224. 1994.

3. Bounous D. I., R. P. Campagnoli, and J. Brown. Comparison of MTT colorimetric assay and triated thimidine uptake for lymphocyte proliferation assays using chicken splenocytes. Avian Dis. 36: 1022–1027. 1992.

4. Breed D. G. J., J. Dorrestein, and A. N. Vermeulen. Immunity to Eimeria tenella in chickens: phenotypical and functional changes in peripheral blood T-cell subsets. Avian Dis. 40:37–48. 1996.

5. Bumstead, J. M., N. Bumstead, L. Rothwell, and F. M. TomLey. Comparison of immune response in inbred lines of chickens to Eimeria maxima and Eimeria tenella. Parasitology 111:143–151. 1995.

6. Choi, K. D., H. S. Lillehoj, K. D. Song, and J. Y. Han. Molecular and functional characterization of chicken IL-15. Dev. Comp. Immunol. 23:165–177. 1999.

7. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. T-cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. 120:2027–2032. 1978.

8. Ishiyama, M., Y. Miyazono, K. Sasamoto, Y. Ohkura, and K. Ueno. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta 44:1299–1305. 1997.

9. Lillehoj, H. S. Immune response during coccidiosis in SC and FP chickens. I. In vitro assessment of T cell proliferation response to stage-specific parasite antigens. Vet. Immunol. Immunopathol. 13: 321–328. 1986.

10. Lillehoj, H. S. Lymphocytes involved in cell-mediated immune responses and methods to assess cell-mediated immunity. Poult. Sci. 70:1154–1164. 1991.

11. Lillehoj, H. S., and E. P. Lillehoj. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Dis. 44:408–425. 2000.

12. Martin, A., H. S. Lillehoj, B. Kaspers, and L. D. Bacon. Mitogen-induced lymphocyte proliferation and interferon production following coccidia infection. Avian Dis. 38:262–268. 1994.

13. Myers, T. J., H. S. Lillehoj, and R. H. Fetterer. Partial purification and characterization of chicken interleukin-2. Vet. Immunol. Immunopathol. 34:97–114. 1992.

14. Ni, J., and D. Hollander. Applications of the MTT assay to functional studies of mouse intestinal intraepithelial lymphocytes. J. Clin. Lab. Anal. 10: 42–52. 1996.

15. Pfohl, J. L., J. B. Hester, V. W. Doelling, R. S. Girardi, J. E. Hutchins, D. L. Murray, C. A. Ricks, and R. M. Poston. Development of a highly quantitative reproducible assay for determination of chicken T cell growth factor biological activity. Poult. Sci. 76:1379–1386. 1997.

16. Rose, M. E., and P. Hesketh. Infection with Eimeria tenella: modulation of lymphocyte blastogenesis by specific antigen, and evidence for immunodepression. J. Protozool. 31:549–553. 1984.

17. Schauenstein, K., A. Globerson, and G. Wick. Avian lymphokines: 1. Thymic cell growth factor in supernatants of mitogen stimulated chicken spleen cells. Dev. Comp. Immunol. 6:533–540. 1982.

18. Tominaga, H., M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hanamoto, K. Suzuki, and M. Watanabe. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal. Commun. 36:47–50. 1999.

19. Wagner, U., E. Burkhardt, and K. Failing. Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the ^{3}H-thymidine incorporation assay. Vet. Immunol. Immunopathol. 70:151–159. 1999.

20. Weichert, H., I. Blechschmidt, S. Schröder, and H. Ambrosius. The MTT-assay as a rapid test for cell proliferation and cell killing: application to human peripheral blood lymphocytes (PBL). Allerg. Immunol. 37:139–144. 1991.

21. Yun, C. H., H. S. Lillehoj, and E. P. Lillehoj. Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24:303–324. 2000.

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